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ORIGINAL PAPER

Technology for cell cycle research with unstressed steady-state cultures

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Abstract A culture system for performing cell cycle analyses on cells in undisturbed steady-state populations was designed and tested. In this system, newborn cells are shed continuously from an immobilized, perfused culture rotating about the horizontal axis. As a result of this arrangement, the number of newborn cells released into the effluent medium each generation is identical to the number of cells residing in the immobilized population, indicating that one of the two new daughter cells is shed at each cell division. Thus, the immobilized cells constitute a continuous, steady-state culture because the concentrations, locations and microenvironments of the cells in the culture vessel do not vary with time. In tests with mouse L1210 lymphocytic leukemia cells, about 10^8 newborn cells were produced per day. This new culture system enables a multiplicity of cell cycle analyses on large numbers of cells assured to be from populations in steady-state growth.

Keywords Steady-state culture · Cell cycle · Continuous culture · Rotating culture

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Introduction

Cell cycle studies often require generation of cells progressing synchronously through the cell cycle. Ideally, the growth state of the culture prior to synchronization should be clearly defined, and the technique employed should not perturb the cells from that state. Most synchronization procedures are initiated on cells presumed to be growing exponentially in a culture flask or bottle (Helmstetter 1969; Grdina et al. 1984; Knehr et al. 1995; Merrill 1998; Davis et al. 2001). However, attainment of true exponential growth in batch cultures of mammalian cells, which may increase only about 10-fold in cell concentration from inoculation to early stationary phase, is problematic (e.g., Skehan and Friedman 1984). Furthermore, batch cultures are not in steady-state. Steady-state can be defined as a stable condition that does not change over time or in

which change in one direction is continually balanced by change in another. In a batch culture system, the cellular environment, including the medium components and biomass, change continuously. As a consequence, it is difficult to unambiguously define the status of the culture upon which cell cycle work is to be initiated.

Even if optimal culture conditions were attainable, such that the cells could be reproducibly specified as being in true balanced growth (Campbell 1957) in a continuous, steady state culture, performing cell cycle analyses on such cultures via synchronization is also challenging. It is well-established that chemical and physiological treatments used to synchronize growth by temporarily arresting cells in a specific stage of the cell cycle disturb the cycle and do not result in true synchronous growth (Gong et al. 1995; Urbani et al. 1995; Cooper 1998). Methods designed to obtain synchronously growing cultures by selecting cells of the same age or size from a growing culture are inherently less likely to disturb cell growth and cell cycle properties. Mitotic shake-off has been considered to be the least disturbing and easiest method for obtaining modest numbers of synchronous cells (Terasima and Tolmach 1963), as long as the state of the cells is not altered by transfer into fresh medium or treatment with mitotic inhibitors to increase cell yields (Merrill 1998; Davis et al. 2001).

Recently we reported the application of a technique used extensively for cell cycle studies on minimally disturbed bacterial cultures to mammalian cells (Thornton et al. 2002). In this technique (commonly called the cellular ''baby-machine''), cells are grown immobilized on a surface such that newborn daughter cells are shed into the culture medium as a consequence of cell division. The released newborn cells have been found to grow synchronously with no overt evidence of growth disturbances. Although this technique has been shown to function well (e.g., Cooper 2002; Helmstetter et al. 2003; Eward et al. 2004a, b; Grover et al. 2004), in all instances the newborn cells were collected during the first few generations of growth after transfer from batch to immobilized growth.

Here we report significant modifications of the cellular baby-machine culture system which enables mammalian cell cycle analyses on cells in long-term steady-state culture.

Materials

A. Cells and culture medium

1. Cells: Mouse L1210 lymphocytic leukemia cells (ATCC, CCL219).[1]
2. Culture medium: Leibovitz L-15 CO_2-independent medium (Invitrogen, 11,415–064) [2] USA supplemented with 2 g l^{-1} dextrose (Sigma-Aldrich,G8769)[3], 2.5 g l^{-1} penicillin-streptomycin solution (Invitrogen, 15,140–122)[2], and 10% fetal bovine serum (Invitrogen, 16,000–044).[2]

B. Chemicals and reagents

1. Concanavalin A (Sigma–Aldrich, C2010).[3]
2. Poly-D-lysine (Sigma–Aldrich, P7886).[3]
3. Dulbecco's phosphate buffered saline (PBS, Invitrogen, 14,040–216).[2]
4. Propidium iodide (Sigma–Aldrich, P4170)[3].
5. RNAase A (Sigma–Aldrich, R6513)[3].

C. Supplies

1. Membrane filter (Millipore, GSWP142).[4]
2. Stainless steel screen (Millipore, YY3014234).[4]
3. Dacron® mesh spacer (Millipore, AP3212450).[4]
4. Polyester support screen (Geotech, 13150016).[5]
5. 150 mm-diameter funnel.
6. Syringes.
7. 1-liter reservoir bottles.
8. Cell culture flasks.

D. Equipment

1. The instrument used in these studies, referred to as the rotating cellular baby machine or

[1] American Type Culture Collection, Manassas, VA, 20110, USA.

[2] Invitrogen, Carlsbad, CA, 92008.

[3] Sigma-Aldrich Co., St. Louis, MO, 63178, USA.

[4] Millipore Corp., Bedford, MA, 01821, USA.

[5] Geotech Inc., Denver, CO, 80207, USA.

RCBM, was fabricated at Johnson Space Center based on the design of a hydrofocusing bioreactor (HFB, Gonda et al. 2003). In the current application, the domed culture chamber in the HFB was replaced by a flat Lexan® disk (165 mm OD, 12 mm thick) with a central 3 mm-diameter opening containing a stainless steel female luer stopcock. The basic instrument can be purchased from Celdyne[6] (rotating bioreactor, HFB-150, with a perfusion coupling kit, RCK-2) and modified as shown in Fig. 1 by replacing the domed chamber with the transparent plastic flat disk, removing the viscous spinner and sealing the back of the culture chamber.

2. Electronic particle counter (Beckman Coulter, model Z2).[7]
3. Flow cytometer.
4. 37°C incubator.
5. Peristaltic pump.
6. Laminar flow hood.

Procedure

Instrument preparation

Prior to an experiment, coat a 142 mm-diameter, 0.22 μm pore size nitrocellulose Millipore membrane filter with a solution of conconavalin A at 5 μg ml^{-1} in 50 ml of PBS. To accomplished this, pour the solution onto a membrane held in a 150 mm-diameter funnel and supported by a Millipore stainless steel screen as described previously (Thornton et al. 2002). Draw the solution through the filter under vacuum at a rate of about 1.0 ml s^{-1}. Then wash the membrane filter by drawing 100 ml of PBS rapidly through the filter. (Poly-D-lysine can also be used at the same concentration.) Place a 124-mm diameter Dacron™ mesh spacer on the membrane before the PBS wash in order to wet it. Assemble the RCBM in a laminar flow hood with the instrument placed vertically so the culture vessel faces up. First, place a 140-mm diameter Geotech® polyester support screen on the culture vessel, followed by the wet membrane with the wet mesh, and then the Lexan® flat plate. No washers or O-rings should be used since they prevent the membrane from lying flat once it has dried. Holes extend through the flat plate and culture vessel at the periphery to accommodate bolts, washers and hexagonal nuts. Assemble the RCBM completely, with the bolts hand-tightened so that the membrane and mesh will dry flat against the plate to prevent the formation of creases, and place it in a 37°C incubator overnight.

Experimental technique

On the day of the experiment, tighten the flat plate firmly against the bioreactor vessel with wrenches to prevent leaks. Fill the culture vessel with culture medium by pumping supplemented L-15 medium from the reservoir with the stopcock closed and ports 1 and 2 (preferably also consisting of female luer stopcocks) open (Fig. 1). For this and subsequent steps in the procedure, the RCBM should be in the configuration shown in Fig. 1. After the vessel is filled, a maximum of 7×10^7 total cells growing exponentially in 300 ml of supplemented L-15 medium in a 150-cm^2 culture flask are introduced into the instrument. Fewer cells may be used, but the yield will be correspondingly lower. Approximately 90% of the cells will remain adhered to the coated membrane. The preferred method for introducing the cells is with a syringe via the exit port of the coupler (a female luer fitting), with the stopcock and port 1 open and port 2 closed. Open the stopcock while the syringe is attached, and close it before the syringe is removed. If left open, a large bubble can form which might loosen cells attached to the membrane. If bubbles form, they are easily removed by opening the vent in the flat plate near the end of cell loading. Remove all bubbles at this time since it is more difficult to do so at a later step. Initiate rotation of the vessel and begin pumping culture medium from a 1-liter, or larger, bottle containing a filtered vent at a rate of 15 ml min^{-1} for the first 2 min to remove

[6] Celdyne Corp., Houston, TX, 77058, USA.

[7] Beckman Coulter Inc., Fullerton, CA, 92834, USA.

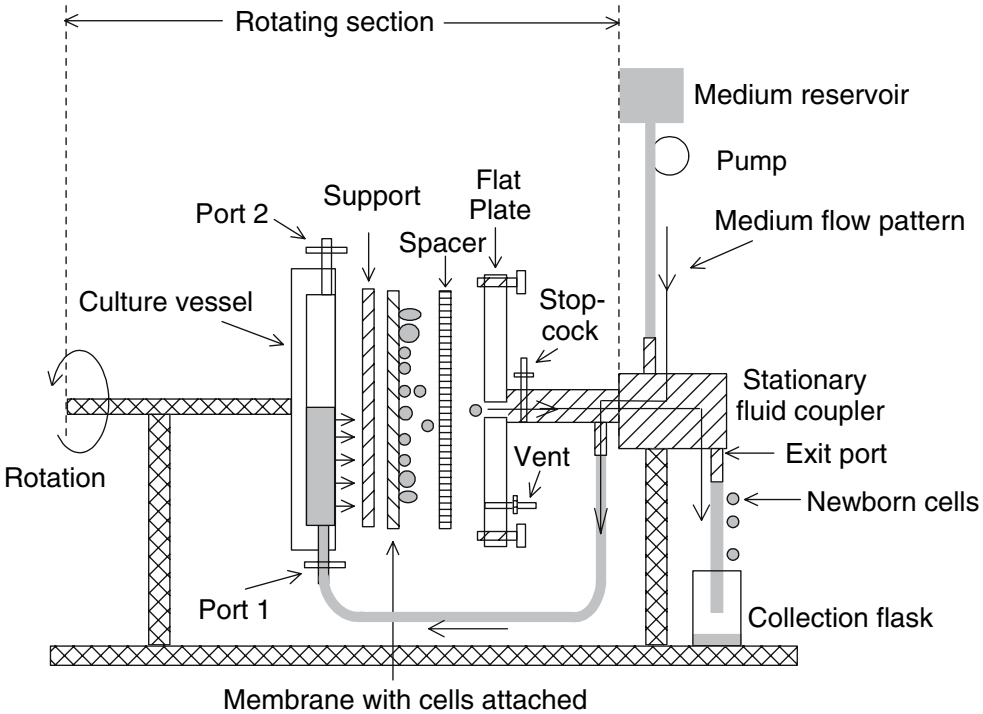

Fig. 1 Schematic representation of the RCBM. Orientation of the RCBM during operation and newborn cell production. See text for details

loosely attached cells and approximately 2 ml min^{-1} thereafter. Set the vessel to rotate at approximately 14 rpm employing a stepping motor connected by two belts to a shaft at the back of the culture vessel. For optimal operation, reduce the culture vessel to one-half full after approximately 30 min of rotation by draining medium from port 2 after disconnecting the tubing and opening port 1. The draining process takes less than 1 min. When the draining is complete, reconnect the tubing to port 1, and commence pumping of culture medium from the reservoir into the vessel with the stopcock and port 1 open, and port 2 and the vent closed. The arrows in the figure indicate the direction of medium flow through the instrument. If additional bubbles form, culture medium can be introduced via syringe as described for loading the cells to remove them through the vent. All procedures should be performed in a 37°C incubator. Cell samples can be collected in 250-ml culture flasks, again at 37°C.

Assay procedures

Cell concentrations and size distributions were determined with a model Z2 Coulter electronic particle counter, containing an aperture tube with a 70 μm diameter orifice. Cell volume determinations were based on instrument calibration with latex spheres of known sizes. Cellular DNA distributions were determined with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) after staining ethanol-fixed cells for 30 min in a solution of 50 μg ml^{-1} of propidium iodide and 100 μg ml^{-1} of RNAase A.

Results

The rotating cellular baby machine (RCBM)

The RCBM consisted of a culture vessel, a support screen, a 142-mm diameter porous membrane with cells attached, a Dacron mesh spacer,

and a flat plate with a central opening, all connected to a motor at one end and a fluid coupler at the other end (Fig. 1). The coupler allowed for the continuous perfusion of culture medium at 2 ml min^{-1} from a reservoir into the culture vessel, through the immobilized population and out an exit port while the culture vessel was rotating. The Dacron spacer created a thin layer (about 0.2 mm) of culture medium between the membrane and the flat plate. As a consequence, the immobilized cells were continuously bathed in culture medium and newborn cells, generated through division within the population, exited the instrument rapidly.

Newborn cell production

Figure 2 shows the concentration of cells released from the RCBM during a long-term experiment with mouse L1210 lymphocytic leukemia cells. The concentration of cells in the effluent was a function of the mitotic cycle age distribution of the culture initially attached to the membrane surface. The theoretical concentration of newborn cells in the effluent is given by the age distribution in reverse, since the first immobilized cells to divide and release one daughter (time 0) would be those that were closest to division at the time of attachment. As younger cells reach the division stage and release a daughter, the concentration of cells in the effluent would increase due to the increased frequency of their parents in the original culture. The theoretical idealized concentration of newborn cells in the effluent is shown in the figure assuming an exponential-phase culture was immobilized on the membrane, and that one newborn cell was released at every cell division in the population. In this case, twice as many cells would be released at the end of the first generation of growth on the membrane because there are twice as many newborn cells as cells about to divide in an exponential-phase population. At this time all of the originally bound cells would have divided once and now begin to divide for the second time, again starting with the oldest cells. Thus, the theoretical cell concentration in the effluent consists of a saw-toothed curve varying 2-fold in each generation. The experimental result mimics the theoretical curve except that the shape gradually decays due to the dispersion in interdivision times of individual cells. As indicated in the figure, the generation time of the cells in the RCBM was approximately 10 h throughout the entire course of the experiment. Approximately 6×10^7 total cells were loaded into the bioreactor and the same number of newborn cells were shed continuously from the bioreactor in each generation. Thus, as far as can be detected, each time an immobilized cell divided one daughter remained attached and the other was released. As a result, the number of cells in the bioreactor and the number of newborn cells released per generation remained constant.

The percentage of eluted cells that were ''newborn'' based on DNA contents and size distributions, as well as mean cell volumes varied little for at least 60 h (Fig. 2). Examples of cell size and DNA distributions at selected times during elution are shown in the upper frames. The percent newborn cells (±SD), estimated by the percent cells in G1 and the percent cells in a discrete small volume interval (Fig. 3), averaged 97.5 ± 0.2% and 96.3 ± 0.7%, respectively, over 60 h. As seen in Fig. 3A, the eluted cells (time 0 h) displayed a narrow size distribution, and this size increased as the cells grew until they progressed through synchronous division at about 9 h and then again at about 18 h. Similarly, the cells in the effluent contained G1 phase DNA content, and they progressed through S phase and G2/M phase DNA contents in each cell cycle (Fig. 3B).

Discussion

The RCBM yielded large quantities of essentially pure newborn cells from a culture in undisturbed, steady-state growth. Steady-state growth was achieved because the number of newborn cells released into the effluent of the bioreactor each generation did not vary for multiple generations. This indicates that one daughter cell was typically shed from the immobilized culture at each division. If that were not the case, and occasionally both daughters remained attached, the total number of newborn cells released into the effluent per generation would have increased in successive generations because the number of

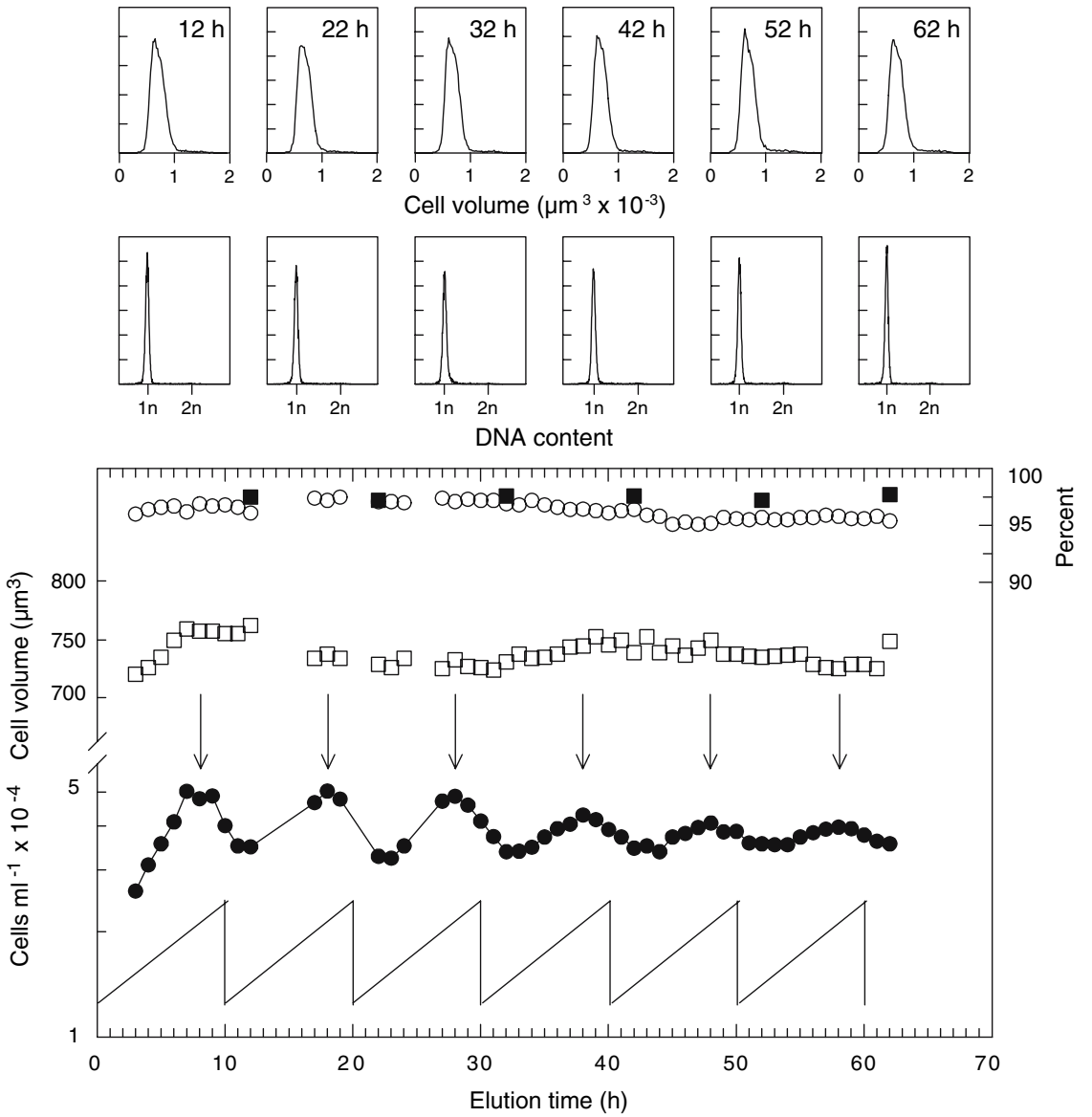

Fig. 2 Theoretical and experimental concentrations of newborn L1210 cells in the effluent from an RCBM. The solid lines reflect the theoretical concentration of cells in the effluent from an immobilized population with an exponential age distribution, assuming the generation time is 10 h and one of the two newborn cells formed at division is always shed from the surface. The number of cells in consecutive samples of the effluent is shown (filled circles) for a membrane-bound culture of L1210 cells growing in L-15 medium at 37°C. Four-min samples were collected from the effluent at the indicated times. Arrows indicate peaks in cell concentration in each generation. Also shown are mean cell volumes (open squares), percent cells that had G1 content of DNA (filled squares) and percent cells that were newborn (open circles), as determined from cell size distributions described in Fig. 3. The upper frames show cell volume and DNA distributions for samples of the effluent collected at the indicated times

cells on the surface would have increased. This was the case for all previous versions of the baby machine for bacteria, yeast and mammalian cells (e.g., Helmstetter 1991; Helmstetter et al. 1992; Thornton et al. 2002). Thus, the cells on the surface, and the released newborn cells, satisfied the definition of steady-state because the condition of the culture did not change over time.

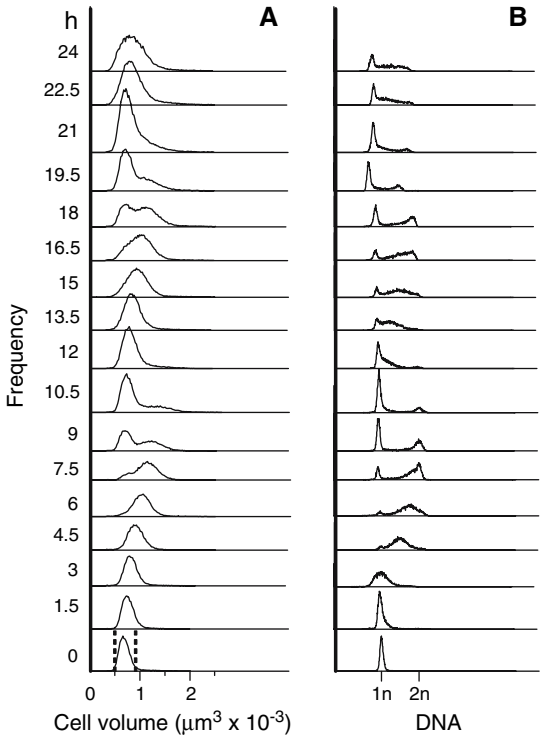

Fig. 3 Cell size and DNA distributions during synchronous growth of mouse L1210 cells. The cells of different ages were obtained by collecting consecutive 40-min samples in 250-ml culture flasks from the effluent of the experiment described in Fig. 2. The first collection started at time 3.5 hr of elution. Each sample was incubated for the indicated time in the cell cycle and size (**A**) and DNA (**B**) distributions were determined. In A, the vertical interrupted lines at time 0 bracket the cells designated as ''newborn''

Neither the number of cells in the culture vessel nor the composition of the fresh medium bathing the cells changed with time, yielding a stable microenvironment for individual cells. Perhaps the continuous culture nature of this system is also the reason that the immobilized cells progressed normally through cytokinesis, which is not necessarily the case in static cultures (Huang et al. 2005). Thus, the key innovation in the current technology is the long-term production of newborn cells from an unstressed, continuous, steady-state culture.

The likely reason for the continuous culture properties of the RCBM is that the released newborn cells were efficiently and rapidly flushed from the surface due to the rotation of the culture vessel and the small, defined volume of medium uniformly bathing the immobilized cells. Based on extensive analysis of the culture technique in various configurations, including time-lapse videos of the behavior of the bound cells, once cells adhere, they appear to remain fixed in place. It is likely that one of the two daughters was released at division because the plane of division was rarely perpendicular to the surface, presumably due to random attachment sites followed by orthogonal division planes (Helmstetter 1997 and unpublished observations), and the lifting action of the medium flow. Thus, not only does this configuration produce steady-state growth but it also optimizes the yield of newborn cells for extended durations due to the near 100% release of newborn cells from the immobilized culture. Although the culture system has only been operated for a week at a time, there was no indication that the concentration and purity of newborn cells in the effluent would change significantly during longer operation. In six experiments performed as in Fig. 2, the average number of cells produced per day varied between approximately 0.8 and 1.2×10^8. A four-day operation enabled collection of about 60 samples of newborn L1210 cells containing approximately 5×10^6 cells each.

The RCBM is a flexible tool for cell cycle research, with two primary applications. Firstly, the technique can be used for collection of synchronous newborn cell samples and performing standard cell cycle examinations during subsequent synchronous growth. Due to the normal broad distribution of interdivision times in growing cultures, a collection for 10% of the generation time does not produce a measurable spread in the quality of synchrony (Grover et al. 2004). Secondly, and perhaps more importantly, the cell cycle properties and behaviors of the steady-state population in the culture vessel can be examined by performing labeling or treatments on the immobilized population and observing the effects in their progeny. These ''backwards'' methods for cell cycle study have been used extensively for studying the bacterial cell cycle and have been described in detail previously (Helmstetter 1969; Cooper 1991). For instance, the cell cycle timing of synthesis of a

molecule can be determined by pulse-labeling the immobilized population and measuring the label on their progeny as they are continuously released. This experimental protocol yields the time of synthesis of the molecule in the cycle in reverse, and was used extensively to decifer the bacterial cell cycle (Helmstetter 1996). Finally, an instrument that yields long-term production of newborn cells from steady-state cultures has potential applications in cell cycle research on cell properties that may change with time, such as replicative aging and differentiation. As described here, the rapid growth and excellent synchrony achievable with mouse L1210 cells make them an attractive model for cell cycle research. However, it is likely that a variety of other cells will function in this culture system as long as they can be adhered to the membrane surface, grow without spreading, and separate within a reasonable time after telophase.

The critically important aspects of the technology relate specifically to the membrane and its arrangement in the system. The recommended membrane type and pore-size, the Geotech® support screen, and the Dacron® mesh should not be altered. During operation, the membrane should lie flat against the mesh and flat plate, with no bubbles other than small ones along the periphery. This requires that no washers or O-rings be used and that the support screen by flexible so that any bubbles that form are easily removed. Other aspects of the procedures are less critical, except that medium pump rates of 1.0 ml min^{-1} or less are insufficient to adequately feed an immobilized population of approximately 6×10^7 L1210 cells.

In conclusion, while the original static ''baby-machine'' technique remains the simplest method for collection of minimally disturbed newborn cells over the first few generations of elution, when the percent eluted L1210 cells that are newborn, based on size distributions, remains 90 ± 2% (Thornton et al. 2002 and unpublished observations), the RCBM enables long-term production of over 96% newborn cells from a culture in steady-state.

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